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Fungal Microbiomes Associated with Green and Non-Green Building Materials

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Abstract

Water-damaged buildings can lead to fungal growth and occupant health problems. Green building materials, derived from renewable sources, are increasingly utilized in construction and renovations. However, the question as to what fungi will grow on these green compared to non-green materials, after they get wet, has not been adequately studied. By determining what fungi grow on each type of material, the potential health risks can be more adequately assessed. In this study, we inoculated green and non-green pieces of ceiling tile, composite board, drywall, and flooring with indoor dust containing a complex mixture of naturally occurring fungi. The materials were saturated with water and incubated for two months in a controlled environment. The resulting fungal microbiomes were evaluated using ITS amplicon sequencing. Overall, the richness and diversity of the mycobiomes on each pair of green and non-green pieces were not significantly different. However, different genera dominated on each type of material. For example, *Aspergillus* spp. had the highest relative abundance on green and non-green ceiling tiles and green composite boards, but *Peniophora* spp. dominated the non-green composite board. In contrast, *Penicillium* spp. dominated green and non-green flooring samples. Green gypsum board was dominated by *Phialophora* spp. and *Stachybotrys* spp., but non-green gypsum board by *Myrothecium* spp. These data suggest that water-damaged green and non-green building materials can result in mycobiomes that are dominated by fungal genera whose member species pose different potentials for health risks.

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mycobiome; fungal growth; ITS sequencing; green product

1. Introduction

Green building materials are derived from recycled or renewable sources (US EPA, 2017). For example, gypsum board, composite board or ceiling tiles can be made of recycled materials. Bamboo flooring is an example of a readily renewable product compared to flooring composed of virgin wood. The building industry and homeowners are utilizing more green-building materials, which should make the built environment more sustainable (Steinemann et al. 2017). However, it is also important to determine if green products are susceptible to more or different fungal growth compared to the products they are replacing (Thatcher and Milner, 2016).

Fungal growth on moisture-damaged building materials can lead to health effects including asthma and other respiratory problems (WHO, 2009). The growth of fungi on green and non-green product pairs has been compared in three earlier studies (Hoang et al., 2010; Huang et al., 2015; Mensah-Attipoe et al., 2015). Hoang et al. (2010) inoculated green and non-green product pairs with either *Aspergillus niger* spores or by allowing the materials to be “naturally” inoculated by placing sterile pieces of each in a home and allowing airborne fungal cells to settle on each piece before testing began. The fungal growth was visually assessed by measuring the area of the surface contaminated by fungal growth. Under either inoculation method, fungal growth was comparable on green and non-green products. Huang et al. (2015) inoculated green and non-green building materials with *Aspergillus brasiliensis* and *Penicillium funiculosum* and found no differences in visually assessed fungal growth on each pair of materials. Mensah-Attipoe et al. (2015) used cultivation and an enzyme bioassay to compare fungal growth on green and non-green product pairs inoculated with three fungi: *Aspergillus versicolor, Cladosporium cladosporioides* or *Penicillium brevicompactum*. No significant differences in the growth of these three fungi on green and non-green building materials were found. However, there are about 1.5 million fungal species (Hawksworth, 2001) and testing each mold separately in such studies is not practical. Therefore, in our tests, we inoculated green and non-green materials with a complex mixture of fungi naturally occurring in indoor dust.

Cultivation has previously been used to assess fungal contamination in field samples (Hyvärinen et al., 2002). This method, however, will detect only fungi that are able to grow on the culture media used. The populations of fungi, or mycobiome, can be studied by using ITS amplicon sequencing (Schoch et al., 2012). Hoisington et al. (Hoisington et al., 2014) used this technology to evaluate the complex mycobiome of a retail store. Using this technology, the identification of fungi in the indoor environment has recently provided new insights into the health effects of previously overlooked fungi, such as the fungal species placed in the genus *Cryptococcus* (Dannemiller et al., 2014). Next-generation sequencing method have also been used to analyze bacterial biomes in building materials (Adamiak et

al., 2017; Laiz et al., 2011). The objective of this study was to evaluate the similarities and differences in the mycobiome developed on green compared to non-green building products.

2. Materials and Methods

2.1 Selection of building materials

Based on consultation with a Leadership in Energy and Environmental Design (LEED) specialist at the U.S. Green Building Council (http://www.usgbc.org), four different types of most commonly used green and non-green building materials were chosen for this study. The green building materials included bamboo flooring (GreenFloors, Fairfax, VA, USA), wheat mineral board (Kirei, Solana Beach, CA, USA), Sheetrock gypsum board (CGC Corporation, Mississauga, Ontario, Canada) and Armstrong Acoustical ceiling tiles (Armstrong World Industries, Hilliard, OH, USA). The main components of the two first building materials are organic material (bamboo and wheat stalks), whereas sheetrock gypsum board contains up to 95% of pre-consumer recycled content and acoustical ceiling tiles which contain up to 82% recycled content which include both pre- and post-consumer waste, as well as materials including recycled newspaper, mineral wool, perlite, jute and cornstarch. The respective nongreen building materials included pine hardwood flooring (BLC Hardwood Flooring, Macon, GA, USA), oriented strand particle board (LP Building Products, Binghamton, NY, USA), conventional gypsum board (Continental Building Products, Herndon, VA, USA) and conventional ceiling tile (SpectraTile, Middlebury, IN, USA).

2.2 Collection and preparation of inoculating dust

Indoor dust containing a complex mixture of naturally occurring fungi was used to inoculate the tested building materials. The dust was collected from five indoor locations by vacuuming floors (Filter Queen Majestic™; HMI Industries Inc., Seven Hills, OH) as previously described (Cho et al. 2006). The collected dust was pooled together and sifted using a 355-μm sieve to ensure homogeneity and exclude large particles. The resulting dust pool was then stored at −20 °C before inoculating the building materials.

2.3 Preparation, inoculation, and incubation of building materials

Each building material was cut into three, identical 25 cm^2 pieces and then gamma irradiated with a minimum dose of 25 kGray to reduce any biological contamination. Each piece was then placed in 20 mL of deionized and autoclaved water to establish a high water activity and for the ease of spreading the dust suspension. The sieved dust was suspended in 0.05% Tween 80 solution to obtain a dust concentration of 50 mg/mL and 0.5 ml of this suspension was inoculated on each building material to provide a final dust load of 1 mg/cm^2.

The inoculated building materials were then placed in eight different, 5.3-liter plastic containers (1 container for each of the 8 building material types) to avoid cross-contamination between building materials as previously described (Seo et al. 2008). The containers were purchased from a local hardware store and disinfected by rinsing with 70% ethanol. The containers were aerated with filter-sterilized air (pore size, 0.2 μm; GE Osmonics Inc., MN) once a day for 10 min at a flow rate of 0.53 liter/min (Murtoniemi et al.

2003). Inoculated building material samples were incubated at room temperature (23 ± 1°C) and a relative humidity of 98% (±1%) to simulate flooding situations, for two months. Two months at high humidity (95%) has been shown to be sufficiently long time for mold growth on several types of building materials(Johansson et al. 2012). The humidity was achieved by placing a saturated K_2SO_4 solution (150 g/liter) at the bottom of each container (Korpi et al. 1998). The temperature and humidity in each container were monitored daily using a humidity-temperature pen (Fisher Scientific Company, Pittsburgh, PA).

2.4 Sample preparation for genomic DNA extraction

After the 2-month incubation period, 10 mm diameter autoclaved cork-borers (Fisher Scientific) were used to scoop out approximately five to six circular pieces (thickness ~ 3 mm) of each building material. The weight of each circular piece varied from 1.5 grams to 4.6 grams, depending on the building material. The bores were then pooled together and placed in a sterile mortar and ground with liquid nitrogen for approximately 2 min or until a fine consistency was obtained as previously described (Ettenauer et al., 2012). The ground material was then transferred to 50 mL falcon tubes and homogenized by manually shaking the powder-like samples by hand. If not used for genomic DNA (gDNA) extraction immediately, the samples were stored in −20 °C.

Genomic DNA was extracted from each building material sample (50 mg) using the MOBIO PowerLyzer® PowerSoil® DNA isolation kit following the manufacturer's instructions (Carlsbad, California). An extract of DNA from each sample was sent to the Research and Testing Laboratory (Lubbock, Texas) for Illumina MiSeq sequencing.

2.5 Illumina MiSeq Analysis

Research and Testing Laboratory performed the Illumina MiSeq sequencing. Forward and reverse fusion primers were used to amplify the ITS1 regions from the DNA sample. The forward primer included the (5′-3′) Illumina i5 adapter (AATGATACGGCGACCACCGAGATCTACAC), an 8-10bp barcode, a primer pad, and the ITS1F primer (CTTGGTCATTTAGAGGAAGTAA). The reverse fusion primer included the (5′-3′) Illumina i7 adapter (CAAGCAGAAGACGGCATACGAGAT), an 8-10bp barcode, a primer pad, and the unlabeled ITS2 primer (GCTGCGTTCTTCATCGATGC). The amplification was performed and visualized as previously described (Kozich et al., 2013; MacIntyre et al., 2015).

The sequences were clustered into OTUs defined at the level of 97% sequence identity using the UPARSE algorithm, after going through de-noising and chimera checking (Edgar, 2013). The de-noising was done to remove short sequences, singleton sequences, and noisy reads. Next, the USEARCH global alignment algorithm and the RDP Classifier against a proprietary database (Research and Testing Laboratory) of high-quality sequences derived from the GenBank (as they existed on December 2014) were used to query the centroid sequence from each cluster.

The output was analyzed using a python program, internally developed at the Research and Testing Laboratory, which assigned taxonomic information to each sequence and then computed and wrote the final analysis files. The data have been deposited with links to

BioProject accession number PRJNA380961 in the NCBI BioProject database ((https://www.ncbi.nlm.nih.gov/bioproject/).

2.6 Statistical analyses

Fungal diversity was assessed using two indices. First, Chao1 richness estimator was calculated to determine the overall richness (i.e., number of distinct organisms present within the samples)

$$S_{chao1} = S_{obs} + \frac{n_i(n_i - 1)}{2(n_2 + 1)}$$

where n_i is the number of operational taxonomic units (OTUs) with abundance i.

Second, Shannon diversity (H') was calculated to assess the overall diversity, which is determined by both richness and evenness (the distribution of abundance among distinct taxa):

$$H' = \sum_{I=1}^{R} p_i ln(p_i)$$

where R is richness and p_i is the relative abundance of the ith OTU.

Rarefaction curves of Chao1 Richness estimate and Shannon Diversity were prepared by rarefying between 100 and 10,000 reads with a step size of 100 reads and 10 iterations per step. Differences in the diversity indices between building material types were analyzed by using an ANOVA. All analyses were conducting in R using the vegan (Oksanen et al., 2013), labdsv (Roberts, 2010), DESeq2 (Anders and Huber, 2010), and phyloseq (McMurdie and Holmes, 2013) packages.

3. Results

The mycobiome analysis resulted in 176,520 OTUs from all green building materials and 281,265 OTUs from all non-green building materials, after quality trimming. Based on this data, the Chao1 richness and Shannon's diversity indices were calculated for green and non-green building materials (Figure 1). The mean richness of fungal taxa on non-green building materials was higher than on green materials, but the difference was not statistically significant. The mean diversity was approximately the same between green and non-green building materials, although green flooring had a much higher Shannon diversity index (1.75) compared to non-green flooring (1.18) (Figure 1).

Figure 2 shows the relative abundance of the fungal genera detected on all building materials by ITS amplicon sequencing. *Aspergillus* spp. had the highest relative abundance on green and or non-green ceiling tiles and green composite boards. However, *Peniophora* spp. dominated non-green composite board and accounted for about 65% of the fungal OTUs. In contrast, *Penicillium* spp. accounted for about 40% and 75% of fungal OTUs on green and

non-green flooring samples, respectively. *Phialophora* spp. followed by *Stachybotrys* spp. dominated fungal growth on green gypsum board but, on non-green gypsum board, *Myrothecium* spp. followed by *Phialophora* spp. dominated (Figure 2). (Both the USEARCH and RDP classifiers were used in this analysis, since the RDP is proprietary. Any differences observed between the two classifiers did not affect the overall results of the analysis.)

Figure 3 shows the double dendrogram for the distance between sample types, based on weighted-pair linkage (y-axis) and associations or co-occurrence of the 25 most common genera on each building material (x-axis). The results demonstrated that the type and classification of the building material were independent of the mycobiome that resulted. For example, the mycobiome from the non-green ceiling tile and green flooring were more closely related than the mycobiomes from their matched pair of materials. In addition, the sequencing data showed that certain genera co-colonized, independent of building material (x-axis). The strongest example of this association was between *Stachybotrys* spp. and *Aspergillus* spp. but *Penicillium* spp. and *Exophiala* spp. with *Cryptococcus* spp. (*Filbasidiella* spp.) and *Candida* spp. to a lesser extent.

4. Discussion

There have been a few previous studies of fungal growth on green and/or non-green building materials (Hoang et al. 2010; Huang et al. 2015; Mensah-Attipoe et al. 2015). Hoang et al (2010), in a component of their study called “Natural Inoculation”, left pieces of sterile “green” building materials to collect settled dust for five days in a home. These pieces were then saturated with water and placed in an approximately 90% relative humidity environment or, in other experiments, non-saturated pieces were placed in the high humidity chamber. In either case, they found that the different types of “green” building materials were colonized at different rates. However, the colonization was only estimated by visual observations and no attempt was made to identify or quantify the resulting fungi.

Huang et al. (2015) were primarily interested in the impact of impregnation of nano-metal particles into green and non-green building materials on fungal growth. However, as part of their study, they inoculated green building materials with one *Aspergillus* spp. and one *Penicillium* spp. and incubated the samples at 85% relative humidity. Under those conditions, they did not see differences in fungal colonization between green and non-green building materials. However, the quantification of fungal growth was only based on visual ratings.

In another study, Mensah-Attipoe et al. (2014) tested the growth of three fungi, one *Aspergillus* spp., one *Penicillium* spp. and one *Cladosporium* spp., on two types of green and non-green building materials. The materials were incubated at 95-97% relative humidity for a number of weeks. Based on culturing and biomass estimates, there were no difference in the fungal growth on these green and non-green building materials.

There are many reasons that our results appear to be different from these previous studies. The previous studies of green and non-green building materials only tested for the growth of

one, a few or airborne fungi. In our study, vacuumed-floor dust, collected from multiple indoor sources, was used to inoculate green and non-green building materials. This dust mixture was used to represent the fungal populations that might accumulate over many months or years, ensuring the presence of a wide spectrum of fungi for potential colonization.

Another difference between our study and previous studies, except for Hoang et al (2010), was that we saturated the pieces of building material with water, before placing them at high relative humidity. However, perhaps the most important difference between our study and previous studies was our use of next generation sequencing to evaluate the fungal mycobiome produced on the building materials.

The mycobiomes on the green and non-green materials produced a richness and diversity of fungal OTUs that were not significantly different but dominating fungal genera were specific to the various materials. These differences in mycobiomes was also demonstrated by the linkages shown in Figure 3. Some apparently unrelated materials, e.g., non-green ceiling tile and green flooring, produced more similar mycobiomes than materials in the same class. In some cases, co-colonization by some genera were independent of building material. Therefore, the distinction between green and non-green building materials may not be the driving force in determining differences in fungal growth, but other factors like microbial competition, chemical composition, moisture and nutrient availability may be more important (Mensah-Attipoe et al.2015).

Although green and non-green materials both supported a rich and diverse mycobiome, these data highlight that the fungal exposures that result may be very different depending on the material. These differences could have health implications, although the identification of the actual species within these genera would help to elucidate the risk of adverse health effects.

Aspergillus spp. OTUs dominated the fungal growth on the green ceiling tile and to a lesser extent the non-green ceiling tile. *Aspergillus* spp. OTUs also dominated the green composite board whereas *Peniophora* spp. dominated the non-green composite bord. The genus *Aspergillus* includes many infectious fungal species (Richardson and Warnock, 2012) but *Peniophora* is a genus that causes white rot of wood and includes some plant pathogenic species (Nagy et al., 2016) but is generally not a source of human pathogens.

Penicillium spp. OTUs were very common on non-green flooring and to a lesser extent on the green flooring samples, but the later was also colonized by *Exophilia* spp., *Candida* spp., and *Cryptococcus* spp.. *Penicillium* species are generally not pathogens (Richardson and Warnock, 2012), but *Exophiala* is a genus that includes black yeasts, some of which are pathogenic species (Cheikh-Ali et al., 2015). *Candida* is a genus whose members are a part of the endogenous human flora but also contains some important human pathogens (Ma et al., 2015; Suhr et al., 2016). The genus *Cryptococcus* (*Filbasidiella*) also includes species that can cause infections, especially for immunocompromised people (Maziarz and Perfect, 2016).

Green gypsum board was dominated by the genus *Phialophora* which contains more than 40 species, most of which are commonly found in soil or on decaying wood but some can also be human pathogens (Brandt and Warnock, 2003). *Myrothecium*, the genus that dominated non-green gypsum board, is a widely distributed genus often found growing on materials made of cellulose but is not a human pathogen (Ahrazem et al., 2000). Although pathogenicity was emphasized in this discussion, almost any fungus is a potential source of allergens, but allergenic potential is highly dependent on the fungal species.

Similar to a previous culture-based study (Hyvärinen et al., 2002), we found *Penicillium* spp. and *Aspergillus* spp. in all building materials. However, we also detected several genera, such as *Myrothecium, Peniophora* and *Crytococcus*, that have not been reported in previous studies that used culture-based analysis of building material samples.

There are several limitations to our study. The mold growth was only tested on building materials derived from one vendor, and other products may behave differently. Another limitation of our study was that the materials were only tested under water-saturated conditions. Hydrophilic genera may be overrepresented compared to genera that are more xerophilic. In the future, we plan to test a more comprehensive set of building materials under a wider set of environmental conditions. However, in spite of these limitations, the results presented demonstrate that, under the conditions tested, an inoculum made of a mixture of dusts can result in differences in the fungal colonization of green and non-green building materials.

The ITS amplicon sequencing analysis itself has several recognized limitations. For example, the use of the 97% cutoff is based upon methods established during the Human Microbiome Project to interrogate 16S amplicon data. This cutoff has not been systematically reviewed or shown to be appropriate for fungal ITS sequencing data. Tonge et al. (2014) found that some fungi cannot be amplified by specific “conserved” primer pairs and suggested that a “multi-region approach be taken for other amplicon-based metagenomic studies”. This was not done in our study and may be a limitation. Also, the sequencing-by-synthesis approach has been reported to be more sensitive to sequence complexity (Tang et al., 2015). Illumina sequencing has also been reported to have biases associated with mold ITS sequencing. For example, primers ITS2 and ITS3 designed to amplify ITS1 and ITS2 regions cause biased amplification towards the Ascomycota and Glomeromycota (Bellemain et al., 2010). Primers ITS1-F, ITS1 and ITS5 have been shown to be biased towards the amplification of the Basidiomycota. Primers specific to amplifying only ITS1 were chosen, which has been suggested to cause the least amount of bias (Bellemain et al., 2010; Bokulich and Mills, 2013). Nevertheless, the next generation sequencing revealed different fungal populations on green and non-green materials that has not been shown in prior studies.

5. Conclusions

The mycobiomes that developed on green compared to non-green building materials were dominated by different fungal genera.

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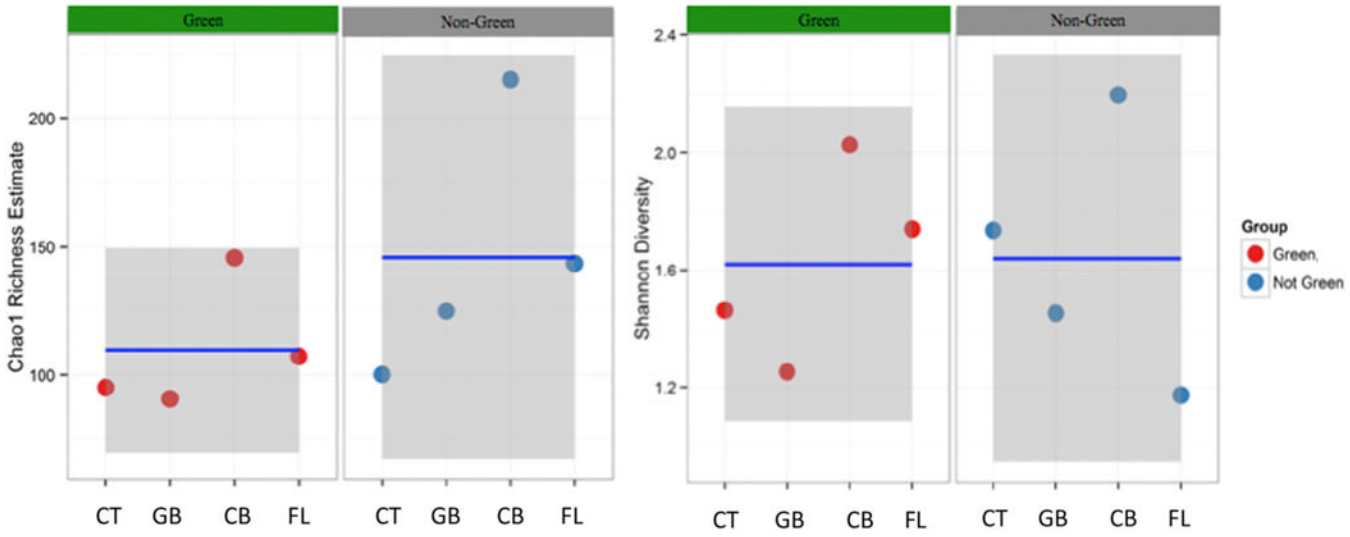

Fig. 1.

Comparison of green and non-green building materials using Chao1 richness and Shannon's diversity indices. The blue line illustrates the mean value of each group (green and non-green). Each circle represents either the richness or diversity value for a building material type. There were no significant differences in Chao1 richness or Shannon diversity between green and non-green materials. (CT=ceiling tile; GB=gypsum board; CB=composite board; FL=flooring)

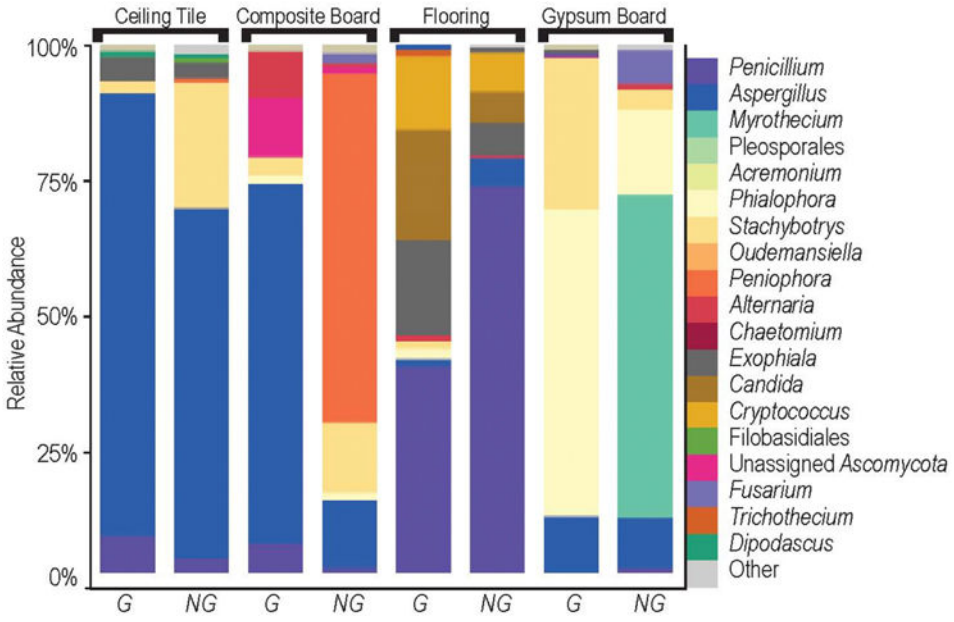

Fig. 2.

Genera detected, in all green and non-green building materials, using ITS amplicon sequencing using RDP-classifier with a confidence threshold of 80%. (Taxons below 1% of relative abundance were categorized as “Other” and depicted by the light gray squares.) (G=green; NG=non-green)

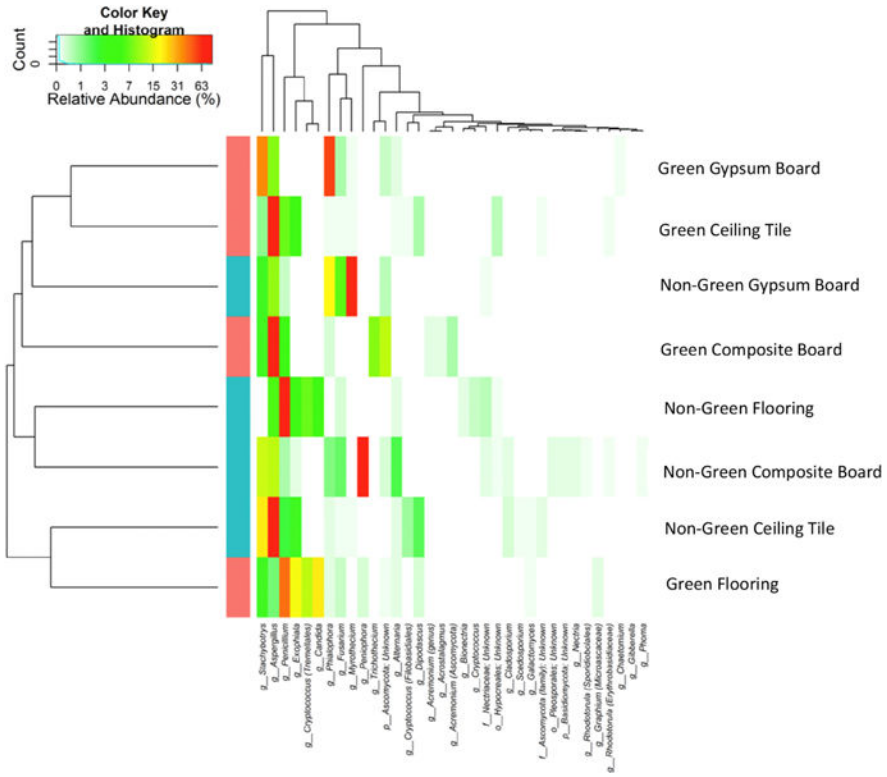

Fig. 3.

Double dendrogram based upon the predominant fungal genera detected, in all green and non-green building materials. The heat map represents the relative percentages of the most abundant fungal genera identified from each sample; white fractions indicate the absence of the fungi. The distance between samples, based on weighted pair linkage, is shown on the y-axis whereas connectedness of the 25 most abundant fungal genera are provided on the x-axis.